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6. AUTHOR(S) H. Shaw Warren				
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13. ABSTRACT (Maximum 200 words) Work over the trimester focused on defining the best LPS binding protein (LBP) peptide sequence, generating peptide-IgG conjugates with this sequence, and assessing the functional ability of these conjugates in buffer and serum. Preliminary work suggested that these, and similar peptides composed of CAP18 and BPI peptides, lost activity in serum compared to buffer. We compared the bactericidal activity of the LBP peptides in serum and buffer, and assessed the ability of conjugates made with these peptides to bind LPS in serum and buffer. We also evaluated the effect of a terminal cysteine on the peptide on peptide activity. We found that that killing was reduced in serum compared to buffer, and that the ability of the LBP peptide-IgG conjugates to bind radiolabeled LPS was reduced in serum compared to buffer. However, the activity of the peptides was dramatically increased with a terminal cysteine. Experiments using ¹⁴ C-peptide confirmed previous estimates that constructs contained 3.9 and 9.5 peptides/IgG.				
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3

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P.I. H. Shaw Warren M.D.
Massachusetts General Hospital
Boston, MA 02114

I. Work Summary

We have continued to focus the majority of our efforts over the past 3-4 months on defining the best LPS binding protein (LBP) peptide sequence, generating peptide-IgG conjugates with this peptide using varying numbers of peptides/IgG and varying peptide-IgG linkers, and assessing their functional activity *in vitro*. At the time of the prior report, we had narrowed the essential sequence of LBP to 86-102 although LBP86-106 also had activity. We also had performed a single experiment in which we found that LBP86-102 coupled with SPDP bound tritiated LPS well in saline, but only poorly in 20% whole blood. Furthermore, earlier experiments, performed with peptide-IgG conjugates based on CAP18, another LPS binding peptide, suggested that some conjugates lost activity during incubation in blood and plasma. Accordingly, a major goal of the last work period was to generate radiolabeled LBP peptides, to couple these peptides to human IgG, and to directly quantitate the number of peptides/IgG and if the peptide was released in serum and blood. These experiments seemed logical and necessary before proceeding on to the animal experiments and are now completed.

A. Progress on Specific Aim #1

This specific aim is almost completed. As noted in the last report, we have identified the minimum essential sequence of LBP needed for binding as including amino acids 86-102. Work in the early part of 1995 focused on identifying the additional advantages of working with peptides with containing amino acids 103-106. Our data suggested that the addition of these three amino acids may confer higher activity in some assays, but that the order of these three amino acids were not important, indicating that part of the activity may be due to enhanced stability of the binding site. We also were puzzled by the finding that several of our peptides were more active in some of our assays with a terminal cysteine that had been added solely for the purposes of coupling. Because of the concern that some of the LPS binding and neutralizing activity that we find was due to dimer formation, we invested considerable effort in blocking the cysteine residues with bulky groups to explore this effect. Although not conclusive, this series of experiments suggested that dimerization of the peptides was not necessary for activity. Because of the experimental design, we were unable to rule out that higher activity could be accomplished with multimers of the peptide.

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We initially had not considered that the LBP peptides might have antimicrobial activity. However, as part of another series of experiments in the laboratory, we utilized one of the LBP86-102C peptides (with cysteine) as a control peptide in a bacterial killing assay. We were surprised to find that this peptide consistently killed a virulent and encapsulated strain of E. coli, E. coli O18K+, at 10 ug/ml. This unexpected and desirable property seemed of considerable interest. Accordingly, we expanded these studies and compared the bactericidal activity of LBP86-102 with and without the cysteine residue. We also included the homologous amino acid sequence of BPI for interest. These findings are shown below, and indicate that LBP86-102 is an antibiotic and that the cysteine residue increases activity.

Bactericidal killing of E. coli O18K+ by peptides in PBS -- % survival after 3 hours

Exp.	Initial, CFU/ml	Final, % bacterial survival				
	Buffer	Buffer alone	conc of peptide (ug/ml)	LBP-cys	LBP-no cys	BPI
Exp. 1	1.8x10e6	4.5x10e6	100	<0.2	11.0	<0.2
	1.8x10e6	4.5x10e6	10	7.5	18.4	48.7
	1.8x10e6	4.5x10e6	1	45.8	20.1	106.2
	1.8x10e6	4.5x10e6	0.1	90.7	20.3	67.9
Exp. 2.	4.0x10e6	3.3x10e6	100	<0.0003	14.3	0.4
	4.0x10e6	3.3x10e6	10	15.4	106.1	101.2
	4.0x10e6	3.3x10e6	1	70.7	82.9	89.9
	4.0x10e6	3.3x10e6	0.1	78.4	68.0	111.0
Exp. 3.	2.9x10e6	2.8x10e6	50	0.003	---	0.5
	2.9x10e6	2.8x10e6	10	6.0	---	33.3

As noted in the last progress report (March 7, 1995), we had found in a single experiment that the LBP peptides seemed less active at binding radiolabeled LPS in 20% whole rabbit blood. Accordingly, we studied the bactericidal activity of the same peptides in 20% whole rabbit blood. Initial experiments were performed in blood anticoagulated with 1 mM EDTA. These experiments indicated that there was a marked difference in bactericidal activity between LBP86-102 with the cysteine and without the cysteine. These experiments are shown at the top of the next page.

Bactericidal killing of E. coli O18K+ by peptides in 20% whole rabbit blood anticoagulated with 1 mM EDTA, diluted in HBSS, % survival after 1 hour

Exp.	Initial, CFU/ml	Final, % bacterial survival				
	Buffer	Buffer alone	conc of peptide (ug/ml)	LBP-cys	LBP-no cys	BPI
Exp. 1	3.5x10e6	3x10e6	50	<0.03	170.0	>3.3
	3.5x10e6	3x10e6	10	30.3	23.7	9.3
	3.5x10e6	3x10e6	2	13.0	6.0	2.0
Exp. 2.	4.8x10e6	1.7x10e5	50	0.2	1176.5	2941.1
	4.8x10e6	1.7x10e5	10	705.9	3294.1	405.9
	4.8x10e6	1.7x10e5	2	188.2	117.6	241.2
Exp. 3.	3.2x10e6	1.2x10e5	50	<0.008	308.3	1000.0
	3.2x10e6	1.2x10e5	10	791.7	1666.7	183.3
	3.2x10e6	1.2x10e5	2	100.0	100.0	83.3
	3.2x10e6	1.2x10e5	0.4	72.5	50.0	38.3
	3.2x10e6	1.2x10e5	0.08	83.3	69.2	35.0

Since divalent cations such as Ca^{++} and Mg^{++} may have a role in the interactions of LPS and other bacterial cell wall components with serum proteins, it was of interest to measure the bactericidal activity of the peptides in the absence of EDTA. Accordingly, we repeated these assays in the absence of any anticoagulant but using teflon tubes (in which there is no coagulation for at least 4-6 hours). These data are shown below.

Bactericidal killing of E. coli O18K+ by peptides in 20% whole rabbit blood, in teflon tubes no anticoagulant, diluted in HBSS, % survival after 1 hour

Exp.	Initial, CFU/ml	Final, % bacterial survival				
	Buffer	Buffer alone	conc of peptide (ug/ml)	LBP-cys	LBP-no cys	BPI
Exp. 1.	2.2x10e6	5.2x10e7	50	>0.2	66.3	7.9
	2.2x10e6	5.2x10e7	10	46.2	96.2	75.0
	2.2x10e6	5.2x10e7	2	55.8	67.7	103.8
Exp. 2.	8.3x10e6	2.5x10e7	50	76.0	144.0	4.0
	8.3x10e6	2.5x10e7	10	152.0	132.0	80.0
	8.3x10e6	2.5x10e7	2	244.0	168.0	136.0
Exp. 3.	2.7x10e6	2.2x10e7	50	26.8	72.7	168.2
	2.7x10e6	2.2x10e7	10	77.3	145.5	109.1
	2.7x10e6	2.2x10e7	2	72.7	86.4	95.5

Together, these experiments indicate that that LBP86-102C, but not LBP86-102, is bactericidal in buffer and in the presence of low divalent cations, but that it is not bactericidal in whole blood in the absence of anticoagulants. Our results are consistent with results obtained with a different cationic peptide in buffer alone published this month (Yomogida S, Nagaoka I, Yamashita T., Involvement of cysteine residues in the biological activity of the active fragments of guinea pig neutrophil cationic peptides, *Infection and Immunity* 63:2344-2377, June 1995).

The role of the cysteine residue in killing by cationic peptides is a new finding. We suspect that the loss of activity in blood in the absence of Ca^{++} depleting anticoagulants is a general phenomenon that may be of considerable importance because study of these and similar peptides in aqueous buffers alone would lead to an overly optimistic interpretation of their activity.

B. Progress on Specific Aim #2

Our encouraging results with the LBP peptides coupled with the work that others were doing in the field with the BPI holoprotein led us to postpone an aggressive search for the active site of BPI. We have previously generated a single peptide based on the homologous sequence of BPI to LBP. This peptide, BPI86-99, binds LPS moderately well and is bactericidal for *E. coli* O18K+ at 50ug/ml as noted in the tables above. We are synthesizing and purifying peptides surrounding this peptide to map the exact sequence that binds LPS with the highest affinity, as written in our original protocol. Although we are interested in bactericidal activity, our primary goal will be to identify sequences that bind LPS, consistent with the concept that protection provided by anti-O IgG correlates with binding affinity and not bacterial killing.

C. Progress on Specific Aim #3

We have worked out the coupling conditions for human IgG and SPDP, SMPT, and SMCC heterobifunctional linkers. We have determined that LBP and CAP18 IgG conjugates are more stable when prepared with SMPT linkers than SPDP linkers. We have made very small test quantities of LBP peptide-IgG and CAP18 peptide-IgG conjugates with SMCC linkers, but have not yet prepared these conjugates in quantities sufficient for testing.

D. Progress on Specific Aim #4

A major goal of the last work period was to exactly determine the number of LBP peptides/IgG. In addition, as of the last report, we had found in a single experiment that the LBP IgG conjugates that we had prepared were extremely active in buffer, but much less active in serum or plasma in binding ^3H -LPS.

To directly assess these issues, we prepared ^{14}C -LPB86-106C by taking resin from the synthesis and exposing it to ^{14}C -acetic anhydride. The peptide was then cleaved from the resin, purified, lyophilized, and counted. This procedure resulted in specific labeling on the NH_2 terminus of the peptide. The specific activity of the peptide was $7.6 \times 10^5 \text{ cpm/mg} = 1.9 \times 10^5 \text{ cpm/mMole}$. We coupled the peptide to 5 mg human IgG using the heterobifunctional linker SMPT aiming for 5 and 10 peptides/IgG. The conjugates were then dialyzed extensively and assessed for total protein and radioactivity to determine the specific

activity of the peptides on the conjugates. These experiments resulted in actual yields of 3.9 and 9.5 peptides/IgG. In previous experiments there was the question if some of the peptide in our preparations was free (not removed from the IgG by dialysis. To study this, we passed the radiolabeled conjugates over molecular sieving HPLC. These studies indicated that the majority of the radiolabel co-eluted with the IgG.

We next compared the ability of the ^{14}C -LPB86-106C to capture ^3H -LPS in PBS, serum, rabbit whole blood, and PBS/BSA by adding 2 ug/ml ^3H -LPS into each solution in the presence of 2-20 ug/ml conjugate. As described in earlier reports and in the original grant protocol, the conjugates (and any bound ^3H -LPS) were then captured by the addition of magnetic anti-human IgG immunobeads. The remaining solutions were then counted for ^{14}C and ^3H , as were the beads, and the percent conjugate and LPS captured were calculated. A representative experiment is summarized below.

Peptides/IgG (conditions)	% ^{14}C capture	% ^3H capture
Sham control IgG (no ^{14}C peptide coupled) (serum)		6
3.9 peptides/IgG (PBS)	90	98
(serum)	77	12
9.5 peptides/IgG (PBS)	97	89
(serum)	86	16

These experiments indicate that the conjugates capture LPS extremely well in PBS, but that binding is markedly diminished in serum. In other experiments, we determined that binding of LPS in physiological concentrations of albumin is also diminished, although not as much as in serum. Although we initially attributed this lack of binding to lack of stability of the conjugates, the experiments demonstrated clearly that this is unlikely because the magnetic beads captured the conjugates, including the ^{14}C radiolabel which is on the amino terminal of the peptide. Since the link of the peptide to the IgG is via the carboxy terminal, a break in the peptide-IgG bond or a proteolytic break in the peptide itself is unlikely. The experiment outlined above documents that the peptide is captured by the anti-IgG beads in both PBS and serum, but the efficacy of ^3H -LPS capture is reduced in serum, even with 9.5 copies of LBP86-106/IgG.

E. Progress on Specific aims #5-7

As noted in the initial grant protocol, we plan to wait for a LBP conjugate that fulfills many of our ideal criteria in vitro before proceeding to the animal experiments. As part of other projects that are not funded by the Navy, and as noted in prior grant reports, we have developed peptide-IgG conjugates from a similar peptide, CAP18. One of these conjugates appears to have quite good binding, neutralizing, and killing activity in serum. Accordingly we are now planning experiments to be done in animals that are similar to those proposed in the initial grant protocol. These experiments will not be performed with Navy funding. However, they are germane in that the experimental protocol

and techniques are similar. We are hopeful that these experiments will pave the way for follow up experiments with LBP peptide-IgG conjugates and BPI peptide-conjugates so that they will run smoothly when the optimal peptide-IgG conjugates are available.

II. New knowledge since the last report

1. LBP86-102 is an antibiotic for encapsulated Gram-negative bacteria in PBS and in blood anticoagulated with Ca^{++} binding anticoagulants. Bactericidal activity is much reduced in blood in the absence of anticoagulants.
2. The activity of LBP86-102 as an antibiotic is dramatically increased by addition of a free cysteine on the carboxy terminal.
3. LBP86-106 conjugates appear to be stable in serum. However, the conjugates ability to capture tritiated LPS is diminished in serum compared to PBS, even with 9.5 peptides/IgG.
4. Use of radiolabeled peptide confirms that our prior calculations regarding number of peptides/IgG were correct.
5. Our findings relating to the behavior of the LBP peptides in whole blood probably has broad implications for the evaluation and study of all LPS binding proteins. Future studies should probably be carried out in whole blood in the absence of calcium binding anticoagulants before proceeding to animal experiments.

III. Technical problems

There have been no technical problems during the last project period.

IV. Budget

As of May, 1995, spending on the grant, as assessed by dollars billed to the Navy, was approximately six months behind our initial anticipated budget. This discrepancy resulted from a combination of two factors. First, although the official start date of grant was January 1, 1994, a quite short notification period before the grant start date and difficulties in arranging and obtaining the generation and purification of the synthetic peptides upon which the project is based resulted in a delay in the start of many of the anticipated experiments for about 4 months. This delay has been carried throughout the spending period. Second, there are approximately \$25,000 of expenses that were ordered or spent but not have not yet been billed. When this sum is subtracted, we estimate that we are approximately 4 months behind in spending, which is where we should be (on budget) if the starting delay in the project is taken into account. I discussed this issue with Mr. Rick Wolfe at the Naval Medical Research and Development Command, Combat Casualty Care, on June 15, 1995. I would like to reaffirm and emphasize that the delay in spending reflects a delay in starting the project and billing that has not yet caught up with actual spending rather than the fact that we are not intensively working on the project.

V. Future Directions

The findings of the last project period that LBP peptides are less

active in blood (as assessed by bactericidal activity and LPS binding activity) is a surprise that has several implications. First, LBP is the sole natural LPS binding protein that is normally found in blood. Accordingly, one might expect that it would retain activity. It is possible that in fact the peptides do retain activity to block the biological activity of LPS and that our bactericidal and LPS binding assays are not adequate to detect this neutralizing effect. This seems unlikely to us, but needs to be ruled out. Accordingly, we will proceed to assess the neutralizing effects of the conjugates on LPS-induced TNF in whole blood. Second, almost all of the assays that have been described with either LBP or BPI have been with blood anticoagulated with citrate or EDTA. Accordingly, it is possible that many of the studies with the holoproteins have been overoptimistic or artifactual. Thus, our finding that calcium binding anticoagulants alter the properties of LPS binding peptides in whole blood may have important implications for studies of all such peptides.

If the LBP86-106 peptide-IgG conjugates fail to neutralize LPS-induced TNF in whole blood, it seems unlikely that this conjugate will be efficacious in animals in its present form. Experiments by our laboratory and others suggest that binding affinity of some peptides can be increased by coupling 1-3 peptides in series. It is also possible that the activity of the conjugates might be increased by adding a longer "link" between the IgG and the peptide. We will try both of these possibilities.

While the above experiments are en route, we will focus on our specific aim #2- identification of the ideal BPI peptide. Because of our extremely encouraging data with the LBP peptides, we have previously focused particularly on LBP. We now will proceed on with the mapping of BPI peptides (already underway) with plans to create BPI-peptide conjugates as initially proposed.

Specific goals for the next work period are:

1. To assess the ability of LBP peptide-IgG conjugates to neutralize LPS induced TNF in whole blood.
2. Design and creation of LBP peptide-IgG conjugates with 2-3 peptides in series and a longer link between the IgG and the peptide.
3. Mapping of the LPS binding site of BPI using the slot blot LPS binding assay.
4. Initial construction and start of testing of BPI peptide-IgG constructs.
5. Finish LBP peptide paper and submit.

VI. Publications submitted or in preparation in the last work period

1. Warren HS, Black KM, Loiselle PL. Range and distribution of natural antibodies to the O-antigen of lipopolysaccharides in human plasma. Submitted to J. Infect. Dis. (funded in part by the preceding Navy grant).
2. Fletcher MA, Kloczewiak M, Loiselle P, Amato S, Black K, Warren HS. TALF peptide-immunoglobulin conjugates that bind lipopolysaccharide. In final preparation for submission for "concise concept" section of J. Inf. Dis. (copy appended)
3. Warren HS, Cavaillon JM, Loiselle P, Ge Y, Black K, Zanzot E, Fitting C, Golenboch D, Vermeulen MW, Ezzell R, Kloczewiak M. Identification of a major LPS binding site of lipopolysaccharide binding protein. In preparation for submission.

*in preparation
for submission
6/22/95*

TALF peptide-immunoglobulin G conjugates that bind lipopolysaccharide

Mark A. Fletcher^{||}, Marek Kloczewiak[†], Paul Loiselle^{||}, Steve Amato^{||}, Kerry Black^{||}, and H. Shaw Warren^{S||*}

Departments of Medicine^S, and Childrens' Service^{||}, Shriners Burns Institute and Massachusetts General Hospital, Harvard Medical School, Boston, MA and Creative Biomolecules[†], Hopkinton, MA.

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Corresponding Author:

H. Shaw Warren
Infectious Disease Unit
Massachusetts General Hospital East
149 13th Street
Charlestown, MA 02129
Tel 617-726-5772
Fax 617-726-5411

Current address for Mark Fletcher:

Mark A. Fletcher, M.D.
Pasteur Mérieux Connaught
3, avenue Pasteur - B.P. 10
92430 Marnes-la-Coquette, France

Abstract

Several peptides mimicking the amino acid sequence of *Tachypleus* anti-LPS factor (TALF) bind LPS with high affinity and some neutralize LPS in vitro and in vivo (J. Infect Dis 1994;170:1490-1497). Two such peptides, TALF29-59 and TALF41-53, were covalently coupled to human IgG via a disulfide bond using the heterobifunctional linker, N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). The resulting peptide-IgG conjugates contained 4-8 moles peptide per mole IgG and were evaluated for the ability to bind and neutralize LPS. Both conjugates bound LPS in a LPS capture Western blot assay. In a fluid-phase radioimmunoassay, half-maximal binding of 5 ug/ml LPS by many different E. coli strains occurred at 50-100 ug/ml of each conjugate. Coagulation of Limulus amoebocyte lysate was only minimally inhibited by 5 ug/ml of each conjugate. Our data suggest that TALF peptide-IgG conjugates bind LPS with high affinity, but only weakly neutralize LPS. These studies provide a first step towards the development of peptide-IgG preparations that might be useful for the treatment of Gram-negative sepsis by binding and clearing LPS.

INTRODUCTION

One strategy for the adjunctive treatment of Gram-negative sepsis has been to develop agents that bind lipopolysaccharide (LPS) released from dying bacteria. Immunoglobulin directed to the O-antigen of LPS binds LPS from the homologous strain with high affinity and is protective in most animal models of LPS or bacterial challenge. However, the large number of bacterial strains that cause clinical disease has limited the practical application of immunotherapy with anti-O immunoglobulin. Monoclonal antibodies directed to common epitopes in the core glycolipid of LPS have been developed to solve this difficulty. Clinical studies of several such antibodies have not been encouraging [Warren, Danner and Munford, 1992], perhaps because these antibodies do not bind with high affinity to LPS [Warren, Amato, Fitting, Black, Loiselle, Pasternack and Cavaillon, 1993].

Non-antibody proteins that bind LPS with high affinity include polymyxin B, bacteriocidal/permeability increasing protein (BPI), cationic protein of MW 18 kD (CAP18), and anti-LPS factors from the amoebocytes of horseshoe crabs, *Limulus polyhemus* (LALF) and *Tachypleus tridentatus* (TALF) [Muta, Miyata, Tokunaga, Nakamura and Iwanaga, 1987]. Each of these proteins bind and neutralize LPS in vitro and protect in animal models of LPS or bacterial challenge. Several of these proteins are being developed as anti-endotoxin agents that could be infused into septic patients in combination with antibiotics. Desirable properties of such agents would include high affinity binding to LPS, a long half-life in the circulation when not complexed with LPS, and absence of immunogenicity or toxicity.

Although considerable work over the last decade has been undertaken to develop agents that neutralize and/or clear LPS from the bloodstream, there is little information suggesting which if either property is more important. Notably, IgG directed to the O-antigen of LPS is highly protective in low doses in animal models, but does not neutralize LPS as assessed by inhibition of LPS-

induced cytokines [Chia, Pollack, Guelde, Koles, Miller and Evans, 1989] or inhibition of LPS-induced coagulation of Limulus amoebocyte lysate [Warren, Novitsky, Bucklin, Kania and Siber, 1987]. This might suggest that clearance and/or phagocytosis is more important for protection than neutralization.

The horseshoe crab anti-LPS factors are single chain peptides of 102 amino acids with one disulfide bridge. Analysis of the crystal structure of recombinant LALF suggests that it is a wedge shaped molecule with three α -helices and a four stranded β -sheet [Hoess, Watson, Siber and Liddington, 1993]. An extended amphipathic loop is hypothesized to be the LPS binding domain [Hoess, Watson, Siber and Liddington, 1993]. Synthetic peptides mimicking sections of the amphipathic loop of TALF bind LPS, inhibit LPS-induced coagulation of Limulus amoebocyte lysate, inhibit LPS-induced induction of cytokines from monocytes, and decrease LPS-induced lethality in mice that are sensitized with actinomycin D [Kloczewiak, Black, Loiselle, Cavaillon, Wainwright and Warren, 1994]. Shorter peptides mimicking half of the loop bind LPS well, but do not neutralize it.

As an initial step towards creating LPS binding peptide-IgG conjugates [Bhattacharjee, Williams, Siber and Cross, 1992] that may function in an analogous manner to anti-O IgG, we covalently coupled peptides that mimic the LPS binding domain of TALF to human IgG. Here we show that this manipulation confers LPS binding activity to murine and human IgG.

METHODS

TALF peptides

Peptides mimicking the binding site of TALF were synthesized using automatic peptide synthesizers (Excel; Milligen/ Millipore, Bedford, MA and PS3; Rainin Instrument, Woburn, MA) as previously described [Kloczewiak, Black,

Loiselle, Cavaillon, Wainwright and Warren, 1994]. Two peptides were selected for coupling to IgG. TALF29-59 was selected because it has the highest binding affinity and neutralized LPS in vitro [Kloczewiak, Black, Loiselle, Cavaillon, Wainwright and Warren, 1994]. TALF41-53 was selected because it is the shortest sequence that retained high binding affinity [Kloczewiak, Black, Loiselle, Cavaillon, Wainwright and Warren, 1994]. Reagents were purchased from NovaBiochem (La Jolla, CA) and from Bachem (Torrance, CA). Peptides were purified to homogeneity by reverse-phase HPLC on 300 Å wide-pore C18 preparative columns. The purity of peptides was checked by analytical HPLC on C18 columns. The amount of peptide was quantified by dry mass.

Coupling of TALF29-59 and TALF41-53 to IgG

Each peptide was synthesized with a non-native cysteine as the carboxy terminal amino acid to facilitate coupling. Peptides were coupled to IgG using the heterobifunctional coupling agent N-Succinimidyl-3-(2-pyridyldithio) propionate (SPDP, Pierce Chemical, Rockford, IL) according to the manufacturers directions and the procedure described by Carlsson [Carlsson, Drevin and Axen, 1978]. Briefly, murine IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) or human IgG (hIgG, Gamimmune N, Cutter Biological, Elkhardt, IN) was diluted to 5 mg/mL with pyrogen-free saline and dialyzed extensively against PBS/EDTA (20 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 0.2% sodium azide, pH 7.2). The IgG was reacted with a 15 molar excess of freshly prepared SPDP (20 mM in DMSO) by adding the coupler in 5 aliquots alternating with gentle vortexing, followed by incubation for 30 minutes at room temperature. Some preparations became turbid and a fine precipitate formed that was removed by filtration on a 0.2µM filter. The SPDP coupled IgG was then dialyzed against acetate buffer (100 mM sodium acetate, 100 mM NaCl, pH 4.5), or desalted against acetate buffer over a G-25 column (Sephadex G-25 Superfine, Pharmacia LKB,

Uppsala, Sweden).

The average number of SPDP groups attached per IgG molecule was determined spectrophotometrically by measuring the absorbance of pyridine-2-thione (Py-2-S) released from the SPDP-coupled IgG upon cleavage of the disulfide bond by addition of reducing agent according to the manufacturers directions and the procedure described by Carlsson [Carlsson, Drevin and Axen, 1978]. A 50 uL aliquot of SPDP coupled IgG was added to 350 uL of carbonate buffer, and A_{280} and A_{343} were measured. The IgG concentration was calculated by the formula: $(\text{IgG}) = A_{280} \times (8/1.38)$. Twenty μL of DTT (50 mM in deionized water) was added. After 10 minutes, A_{343} was measured again. The molar concentration of released Py-2-S was calculated according to the formula: $[\text{Py-2-S}] = (\text{change in } A_{343}) \times (8/8080)$. The calculated IgG concentration was corrected for A_{280} attributable to SPDP according to the formula: $(\text{IgG, corrected}) = (\text{IgG}) - ([\text{Py-2-S}] \times 5100)$. Finally, the molar ratio of SPDP groups to hIgG in the SPDP-hIgG was determined by the formula: $\text{mol Py-2-S/mol IgG} = [\text{Py-2-S}] \div (\text{hIgG, corrected}) \times 0.15$.

TALF peptides, dissolved in pyrogen-free water, were added to the SPDP coupled IgG at a molar excess (1.2 to 5-fold) relative to the number of SPDP groups per IgG. The reaction mixture was incubated overnight at room temperature with gentle rocking. The resulting product was extensively dialyzed against PBS (pH 7.2) to remove uncoupled peptide. Since one Py-2-S group is displaced for each peptide bound, the Py-2-S/IgG ratio was calculated again. An estimate of the number of TALF peptides bound to the IgG was calculated by establishing the difference in Py-2-S/IgG before and after reaction with the peptide.

LPS and bacteria

LPS from E. coli O111:B4, E. coli O113, and S. typhimurium were purchased (List Biochemical, Campbell, CA). Intrinsically radiolabeled LPS from E.

coli O4, O6, O25, and O75 were made by growing each strain in tritiated acetate followed by extraction with hot phenol as described [Warren, Amato, Fitting, Black, Loiselle, Pasternack and Cavaillon, 1993].

Western blot capture assay

Proteins were separated by SDS-PAGE and transferred to nitrocellulose (200 mA, constant current, 1 hour). Nitrocellulose was blocked in a solution of 1% skim milk in 150 mM NaCl, 50 mM TRIS, 0.1% Tween-20, pH 7.5 (TTBS) for 1 hour, washed (TTBS, 10 minute X 3), and then incubated with E. coli O111:B4 or S. typhimurium LPS (10 ug/mL) in 150 mM NaCl, 50 mM TRIS, pH 7.5 (TBS) for 1 hour. Following washes, nitrocellulose was incubated with lapine anti-E. coli O111 LPS or anti-S. typhimurium antiserum (1:500 in TTBS) for 1 hour, followed by washes. Next, the nitrocellulose was incubated with biotin-conjugated anti-rabbit IgG (Vectastain, Vector Laboratories, Burlingame, CA, 1:240 in TTBS) for 30 minutes, washed, and then exposed to avidin-biotinylated horseradish peroxidase complex (Vectastain, 1:120 in TTBS) for 30 minutes. After a final wash, peroxidase substrate was added (2 mL of 3 mg/mL 4-chloro-1-naphthol, 8 mL PBS, 10 uL 30% H₂O₂). The reaction was stopped after 30 minutes by repeated rinsing with distilled water. Some blots were developed directly for the presence of human IgG by incubating the blot with biotin conjugated anti-human IgG (1:240, Vectastain), followed by incubation with avidin-biotinylated horse-radish peroxidase complex (1:240 in TTBS, Vectastain). The blots were then developed as above.

Fluid-phase radioimmunoassay

This assay was performed as previously described [Warren, Amato, Fitting, Black, Loiselle, Pasternack and Cavaillon, 1993]. Briefly, serial dilutions of the TALF peptide-hIgG conjugates were incubated with 5 ug/mL ³H-LPS

in PBS buffer containing 1 mg/mL bovine serum albumin for 2 hours at 37°C. Samples were then placed in an ice bath for 15 minutes, after which an equal volume of iced saturated ammonium sulfate was added and the resulting mixture was incubated for another 15 minutes. Precipitated protein (together with bound ³H-LPS) was collected by centrifugation (12,000 X g, 15 minutes). Under these conditions over 95% of the IgG and less than 5% of the unbound LPS are precipitated. The supernatants were saved and the pellets washed repeatedly, after which ³H-LPS was measured in each using a β-scintillation counter (Packard ADD HERE). Binding is expressed as the percentage of total counts recovered according to the equation: CPM pellet / (CPM pellet + CPM supernatant) X 100.

LAL neutralization assay

A spectrophotometric Limulus amoebocyte lysate (LAL) assay was utilized as previously described [Warren, Amato, Fitting, Black, Loisel, Pasternack and Cavaillon, 1993]. Briefly, 50 ul of a solution of 5 ug/ml of each peptide-IgG conjugate in pyrogen-free saline were incubated with dilutions of LPS for 30 minutes at 37°C in a 96 well microtiter plate. One hundred ul of LAL (Associates of Cape Cod, Falmouth, MA) were then added and the plate was incubated at 37°C for an additional 60 minutes. Coagulation of the LAL was measured spectrophotometrically at 405 nm in an automated ELISA reader. Coagulation of the LAL in the presence of conjugate is compared to coagulation of the LAL in saline alone.

Bactericidal assays

E. coli O16, E. coli O18K+, and E. coli O25 were grown to mid log-phase and washed with saline. Aliquots of bacteria diluted to 10⁶ colony forming units/mL were then added to saline, dilutions of each TALF peptide-hIgG conjugate, or hIgG and incubated (1 hour, 37°C). Serial 10-fold dilutions of each solution in normal saline were then plated on tryptic soy agar and incubated

overnight at 37°C, after which colonies were counted and percent killing determined. Killing of 99% or greater is considered to be evidence of bactericidal activity.

RESULTS

Peptide-IgG conjugates

Multiple early experiments indicated that it was possible to couple each peptide to murine IgG. However, we quickly turned to human IgG because of the high cost of murine IgG that was sufficiently pure and because we were unable to purchase murine IgG that was free of LPS as assessed by LAL.

Two lots of TALF29-59-hIgG were prepared and four lots of TALF41-53-hIgG were prepared. A fifteen-fold molar excess of SPDP to hIgG resulted in an average of 4 to 8 moles of Py-2-S per mole hIgG, indicating that there were 4-8 potential peptide binding sites per hIgG. Peptides were added in greater than an equimolar excess to the Py-2-S to ensure complete replacement of the Py-2-S by peptide.

Binding Assays

The ability of the conjugates to bind LPS was assessed by two methods. In the first method, TALF-hIgG conjugates were blotted onto nitrocellulose and the ability to capture LPS was subsequently detected with lapine anti-LPS IgG specific for the O-antigen of LPS. Both TALF29-59-hIgG and TALF41-53-hIgG captured LPS, whereas normal hIgG and sham SPDP-coupled hIgG failed to capture LPS. Representative SDS-PAGE gels and blots are shown in Figure 1. A small proportion of higher molecular weight proteins was present in the TALF-hIgG on the SDS-PAGE gels and immunoblots that was not present in the normal hIgG. As expected, reduction of the TALF-IgG conjugates with 2-mercaptoethanol cleaved the disulfide bond coupling the peptide to the IgG and resulted in loss of

binding by IgG heavy or light chains.

TALF29-59-hIgG and TALF-41-53-hIgG bound tritiated LPS from numerous heterologous strains of E. coli in a dose dependant manner in the fluid phase radioimmunoassay (Figure 2). There was no substantial difference in binding between the two conjugates.

LAL neutralization assays

Fifty percent LAL activation consistently occurred at approximately 10 pg LPS/mL in 5 ug/ml unconjugated IgG. It required 2 to 3-fold higher concentrations of LPS for equivalent coagulation of LAL in the presence of 5 ug/ml of either TALF-IgG conjugate (data not shown).

Bactericidal assays

Neither TALF-hIgG conjugate was bacterocidal for E. coli O16, E. coli O18K+, or E. coli O25, even at an IgG concentration of 1.0 mg/ml.

DISCUSSION

The goal of our experiments was to couple synthetic peptides based on the LPS binding site of TALF to IgG in order to confer LPS binding properties to human IgG [Bhattacharjee, Williams, Siber and Cross, 1992]. Our rationale for creating such a peptide-immunoglobulin conjugate is that infusion of IgG directed to the O-antigen of LPS is highly protective to challenge with LPS from the same strain in most animal models of Gram-negative sepsis. Accordingly, a preparation that would bind to the LPS from multiple Gram-negative strains represents a first step towards the development of a therapeutic immunoglobulin preparation.

We have previously studied synthetic peptides mimicking the LPS binding sequence of TALF for their ability to bind and neutralize LPS. We found that the peptide that had the best binding activity, TALF29-59, also neutralized LPS-

induced coagulation of LPS and protected sensitized mice against LPS-induced lethality [Kloczewiak, Black, Loisel, Cavaillon, Wainwright and Warren, 1994]. This peptide sequence is contained within an amphipathic loop that extends from the rest of the wedged shaped structure of TALF as assessed by X-ray crystallography [Hoess, Watson, Siber and Liddington, 1993]. The sequence of the shortest peptide that bound LPS with high activity, TALF41-53, is contained within half of this loop. This synthetic peptide did not neutralize LPS in vitro. We chose to couple both peptides to IgG.

Although both conjugates bound LPS equally in the solid and fluid phase assays, neither inhibited LPS-induced coagulation of LAL at 5 ug/ml. We have previously shown that the TALF29-59 peptide neutralizes in this assay in approximately the same peptide concentration that we estimate is present in the conjugate at this IgG concentration [Kloczewiak, Black, Loisel, Cavaillon, Wainwright and Warren, 1994], suggesting that the neutralizing ability of this peptide may be in some way compromised by the coupling. In the fluid phase radioimmunoassay, both conjugates bound 100- to 1000-fold better than what we previously found for two monoclonal antibodies directed to lipid A, although this binding is about 5-fold less than a monoclonal antibody directed to the O-antigen [Warren, Amato, Fitting, Black, Loisel, Pasternack and Cavaillon, 1993].

The properties by which anti-O IgG confers protection are unknown, although anti-O IgG does not neutralize LPS in vitro. Two good possibilities include increased clearance of LPS or bacteria and increased phagocytosis. We plan to evaluate how the TALF-hIgG conjugates perform in these assays and in models of LPS or bacterial challenge when larger quantities of the conjugates are available.

Legends to figures

Figure 1. SDS-PAGE and LPS capture Western blot of identical gels for TALF41-53-hIgG (A panels) and TALF29-59-hIgG (B panels). Panel A1 is 7.5% SDS-PAGE with 1 μ g of the following IgG preparations added to each lane: lane 1, normal hIgG; lane 2, Py-2-S-hIgG; lane 3, TALF41-53-hIgG. Panel A2 is capture Western blot of identical gel as A1 that was incubated with 10 μ g/ml E. coli O111 LPS and developed with rabbit anti-E. coli O111 IgG as described in Methods. Panel B1 is 10% SDS-PAGE with 1 μ g/ml of the following IgG preparations added to each lane: lane 1, normal hIgG; lane 2, normal hIgG, reduced; lane 3, TALF29-59-hIgG; lane 4, TALF29-59-hIgG, reduced. Panel B2 is a Western blot of identical blot of gel in B1, incubated with biotin conjugated anti-human IgG and developed as described in Methods. Panel B3 and B4 are Western blots of identical gel as B1, incubated with 10 μ g/ml E. coli O111 LPS and then rabbit anti-E. coli O111 IgG (B3) or S. typhimurium LPS and then rabbit anti-S. typhimurium IgG (B4) prior to developing blot with biotin conjugated anti-rabbit IgG as described in Methods.

Figure 2. Fluid phase radioimmunoassay of TALF peptide-hIgG conjugates. In A, varying concentrations of TALF41-53-hIgG (solid circles) and TALF29-59-hIgG (solid squares) or normal hIgG from same preparation of TALF41-53-hIgG (open circles) and TALF29-59-hIgG (open squares) were incubated with 5 μ g/ml 3 H-LPS from E. coli O25 prior to addition of saturated ammonium sulfate. In B, varying concentrations of TALF41-53-hIgG (solid symbols) or sham-coupled hIgG from same preparation were incubated with 5 μ g/ml of 3 H-LPS LPS from E. coli O4 (crosses), E. coli O6 (diamonds), E. coli O25 (circles), and E. coli O75 (squares) prior to addition of saturated ammonium sulfate.

